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HMG-I(Y) proteins are mammalian architectural transcription factors. They have been demonstrated to be necessary for the transcriptional regulation of a number of genes, including the genes associated with tumorigenesis and metastasis. It was proposed in the original grant that over-expression of HMG-I(Y) proteins in breast cancer cells will fundamentally influence the expression of genes that are involved in metastatic invasion, migration, angiogenesis and colonization. In the first year of study, the preliminary results support the hypothesis. They include (I) over-expression of HMG-I(Y) in the human breast cancer cell line MCF-7 leads to higher tumorgenic potential, (II) HMG-I(Y) proteins can physically interact with promoter regions of the matrix metalloprotease MMP-3. In addition, new findings have been obtained that HMG-I(Y) proteins can be phosphorylated by protein kinase C in vitro and phosphorylation of HMG-I(Y) proteins can be induced by the mitogen PMA in the human breast cancer cell line Hs578T in vivo. These results indicated that HMG-I(Y) proteins indeed may play an important role in tumor metastasis.

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INTRODUCTION

High mobility group proteins I and Y are mammalian architectural transcription factors that bind to the minor groove of A-T rich DNA (1). They are the products of a signal gene, the HMG-I(Y) gene, derived by alternative RNA splicing. HMG-I(Y) proteins are known specifically interact with several known sequence-specific transcription factors, such as NF-κB, Elf-1, Ap-1, Sp-1 and Oct-1,2 and 6 (2, 3, 4, 5, 6, 7). HMG-I(Y) proteins have also been demonstrated to regulate a growing number of genes in either a positive or a negative fashion in vivo, such as ICAM, VCAM, E-selectin, IL-2Rα and TNF (8, 9, 10, 4, 11). It has been postulated that HMG-I(Y) proteins act as "molecular glue" to promote the cooperatively binding of transcription factors and organize the stereospecific enhanceosomes that are required for gene regulation in vivo (4, 12).

The expression of HMG-I(Y) is correlated with cancer. A body of evidence has shown that the level of HMG-I(Y) is significantly higher in neoplastically transformed cells, metastatic tumor cells, and in undifferentiated embryonic cells than that in normal cells (1). Therefore, it has been proposed as a marker for tumor progression and metastatic potential (13). As shown in **Table 1**, many genes that are associated with tumor progression and metastasis contain multiple and long stretches of AT-rich sequences that are potential binding and regulatory sites for HMG-I(Y) proteins. Among these genes, E-selectin, ICAM, VCAM and TNF-α have already been experimentally demonstrated to be transcriptionally regulated by HMG-I(Y) in vivo (8, 9, 10, 11).

The funded proposal is to define the role of HMG-I(Y) in tumor metastasis. The experimental strategies are: (I) over-expression of HMG-I(Y) in non-metastatic tumor cell lines and over-expression of dominant negative HMG-I(Y) or anti-sense HMG-I(Y) in highly metastatic tumor cell lines; (II) monitor the characteristic changes of these transfectants on the expression of genes, such as adhesion molecules, matrix metalloproteases, which are involved in metastatic invasion, migration, angiogenesis and colonization. During the first year of the funding period, the initiating work has been done as described in the Statement of Work.

RESULTS AND DISCUSSION

(1) Construction and characterization of mammalian expression vectors, which contain sense, antisense or dorminant negative human HMG-I(Y) genes

The sense and antisense cDNA segments were subcloned from previously isolated cDNA of human HMG-I and HMG-Y (14). These cDNA segments were inserted into the selectable mammalian expression vector pcDNA3.1-Zeo that was purchased from Invitrogen, San Diego, CA. All constructs were confirmed by DNA sequencing.

The dominant negative mutated human HMG-I was generated by site-directed mutagenesis. The mutation was made by replacing 4 prolin residues to alanines at DNA binding domains II and III in HMG-I(Y) proteins (14). The designated dominant

negative human HMG-I should lack DNA binding ability but retain its ability of interaction with other transcription factors. The mutated HMG-I was expressed in E.coli and purified by HLPC. The DNA binding activity of the mutated HMG-I was determined in vitro by electrophoretic mobility shift assay (EMSA) and the result showed that the DNA binding activity was eliminated by mutation (**Figure 1**). The ability of interaction with other transcription factors NF-κB and Ap-1 will be tested by protein-protein cross-linking assay in vitro (15).

In vivo effects of antisense and dominant negative HMG-I(Y) have been examined by using the IL-2 gene expression system (containing HMG-I(Y) binding site) in Jurket cells (16). The preliminary experiment showed that both antisense and dominate negative mutated HMG-I(Y) inhibit the IL-2 promoter activity after PMA stimulation (**Table 2**).

Table 2. Antisense of HMG-I and mutated HMG-I can inhibit the activity of IL-2 promoter in response of PMA stimulation in Jurket cells.

Cotransfection	Luciferase activity*
Il-2-promoter-luci and empty vector	**5000-5920**
Il-2-promoter-luci and antisense of HMG-I	**3000-3080**
Il-2-promoter-luci and dominant negative HMG-I	**2640-2800**

* from two independent experiments

(2) Expression of HMG-I(Y) proteins and tumor metastasis in MCF-7 and P-/P+ cell systems

The non-metastatic human breast cancer cell line MCF-7, which expresses a low level of HMG-I(Y) (**Figure 2**), has been transfected with the mammalian expression vector encoded with HMG-I alone, HMG-Y alone, or co-transfected with both of the vectors. The pools of transfectants were selected by the antibiotic Zeocin. After the passage of 25 generations, the Zeocin-resistant MCF-7 cells were examined by soft agar assay. As shown in **Figure 3**, over-expression of HMG-I or HMG-Y or both in MCF-7 cells leads to considerably more, and larger colonies than those transfeced with empty vector in soft agar. The metastasis potential will be tested in nude mice in vivo and invasion assay in vitro.

The highly-metastatic MCF-7/ PKC-α cells, which are stable-transfected with a cDNA coding for protein kinase C-α (PKC-α), were provided by D. K. Ways, East Carolina University of School of Medicine, NC (17). The protein levels of HMG-I(Y) in MCF-7/ PKC-α cells and parental MCF-7 were examined by Western blot. The result demonstrated that HMG-I(Y) proteins in MCF-7/ PKC-α are about five times higher than those in MCF-7 cells (**Figure 2**). Therefore, the MCF-7/ PKC-α cells will be used as target cells to transfect antisense and dominant negative HMG-I(Y) in order to test the role of endogenous HMG-I(Y) on tumor metastasis.

A murine model system of epidermal cell lines P-, P+ and Tx, which were provided by N. Colburn, National Cancer Institute, will be adopted into this study. These cell lines are all derived from the epidermal JB6 cell line and different in tumorigenic and metastic potential (18, 19). The P- cells are non-tumorgenic, even

when exposed to mitogen 12-O-tetradecanoyl phorbol acetate (PMA). The P+ cells are also non-tumorgenic but in contrast to the P- cells, they become tumorgenic after exposed to PMA. The Tx cells are stably transformed P+ cells (19). The data from Western blot revealed that the ratios of the basal level of HMG-I protein in P-, P+ and Tx are about 1:2.5:4.5 and and the basal level of HMG-Y protein in these cells are about 1:1.5:4.5, respectively (data not shown). As shown in **Figure 4**, kinetic study in response to PMA indicated that HMG-I(Y) proteins in P- cells peaked at 12 hrs and then decreased at 24 hrs. However, HMG-I(Y) protein in P+ cells increased continually to 24 hrs after exposed to PMA.

(3) Regulation of metastasis-associated stromelysin (MMP-3) gene by HMG-I(Y)

The regulatory effects of HMG-I(Y) on down-stream genes associated with tumor metastasis were first tested by electrophoretic mobility shift assay (EMSA) on the promoter and enhancer regions of stromelysin (matix metalloprotease type-3, MMP-3). The results, as shown in **Figure 5,** demonstrated that HMG-I(Y) proteins specifically bind to AT-rich sequences in promoter and enhancer regions of the stromelysin. Further experiments are needed to determine the expression of stromelysin and other metastasis-associated genes.

(4) Phosphorylation of HMG-I(Y) proteins by protein kinase C in vitro and in vivo

The phosphorylation of HMG-I(Y) proteins may regulate functions of HMG-I(Y) and in turn regulate other down-stream gene expression. Our preliminary result indicated that protein kinase C (PKC), which is the one of the major protein kinases in controlling cell proliferation and differentiation, can phosphorylate HMG-I(Y) proteins in vitro (**Figure 6**). In addition, both HMG-I and HMG-Y proteins are phosphorylated following stimulation with PMA/ionomycin in the human breast cancer cell line Hs578T in vivo (**Figure 7**).

Table 1: Metastatic Genes with Potential HMG-I(Y) Binding Sites in Their 5' Promoter/ enhancer regions

Gene	Metastatic Stage	Relative No. HMG-I(Y) Sites	Other Factor Binding Sites
PDGFR	Invasion/migration	++	NF-kB, Sp1, PEA3, AP-1, AP-2, GATA
ER	Invasion/migration	++	AP-1, SRE, TPA
EGFR	Invasion/migration	++	Sp1, TC-rich
c-erb-B2	Invasion/migration	++++++	Sp1, AP-1
Vimentin	Cytoskeleton/ Intermed. Filaments	++++++	AP-1, NF-kB
E-cadhedrin	Invasion/migration	+++	Sp1, AP-1
E-selectin	Invasion/migration	++++	NF-kB, ATF-2, AP-1, CRE/ATF
Integrin-β3	Invasion/migration	++++++	Sp1, AP-1
Integrin-α II	Invasion/migration	++++++	C/EBP, NF-kB, AP-1, AP-2, PEA1, PEA3, Sp1, GATA, ATF, H1 sites, H4 sites, E2A
ICAM-1	Invasion/migration	++++++	NF-kB, Sp1, AP-1, AP-2 ATF-2, IRE
VCAM-1	Invasion/migration	++++++	NF-kB, Sp1, AP-1, AP-2, ATF-2
type IV-Collagenase (MMP-9)	Invasion/migration	++++++	AP-1, Sp1, TIE, TRE, CA-rich sequence
uPA	Invasion/migration	++++++	AP-1, Sp1, NRE
Plasminogen	Invasion/migration	++++++	AP-1, Sp1
Matrilysin (MMP-7)	Invasion/migration	++++++	AP-1, PEA3
Stromelysin (MMP-3)	Invasion/migration	++++++	AP-1, RARE, TPA-RE, GRE
Scatter Factor	Migration	++++	Sp1, SRE
FGF-7	Invasion/migration Angiogenesis	++++++	AP-1, AP-2, GATA, PEA3
TGF-β	Angiogenesis	+++	CREB, ATF-2, AP-1, AP-2
TGF-α	Angiogenesis	++	Sp1, AP-2
IFN-β	Angiogenesis	+++	NF-kB, ATF-2
TNF-α	Angiogenesis	+	NF-kB, Sp1, TC-rich seq

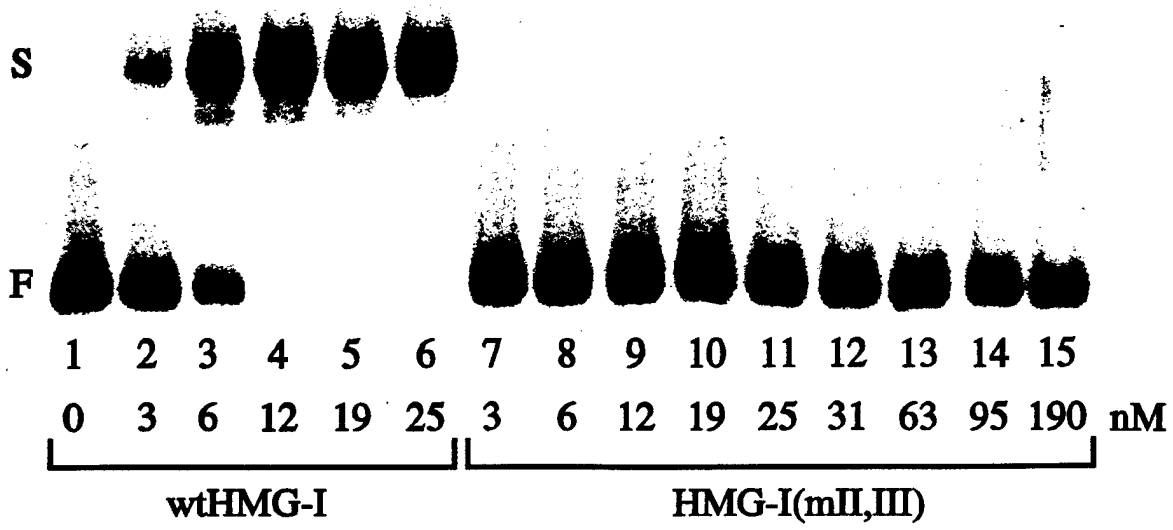

Figure 1: Point mutations in the HMG-I DNA binding domain II and III (mII, III) inhibit DNA binding. Electrophoretic mobility shift assay (EMSA) was performed by using ^{32}P-labeled DNA from the IL-2 promoter region. The DNA was incubated with HLPC-purified wtHMG-I or mutated HMG-I (mII, III) for 20 min at room temperature, followed by eletrophoresis on 4% polyacramide gel. Lane 1, free DNA; Lane 2-6, increasing concentrations (nM) of wild-type HMG-I binding to DNA; Lane 7-15, increasing concentrations (nM) of mutated HMG-I binding to DNA.

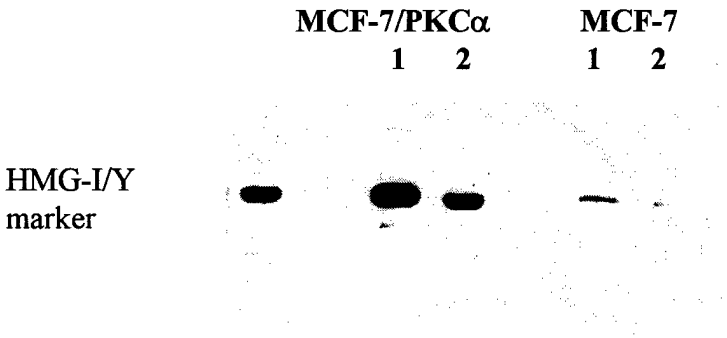

Figure 2: HMG-I(Y) proteins are expressed at a low level in non-metastatic MCF-7 cells but at a high level in highly metastatic MCF-7/PKCα cells. Total cellular proteins were isolated from MCF-7 and MCF-7/PKCα, run on SDS-PAGE, and then transfered onto cellulose membrane, followed by Western blot with a polyclonal antibody against human HMG-I(Y). In each case, Lane 1 contains 12 μg, and Lane 2 6 μg, of protein. A recombinant HMG-I(Y) protein marker was run on the left of the gel.

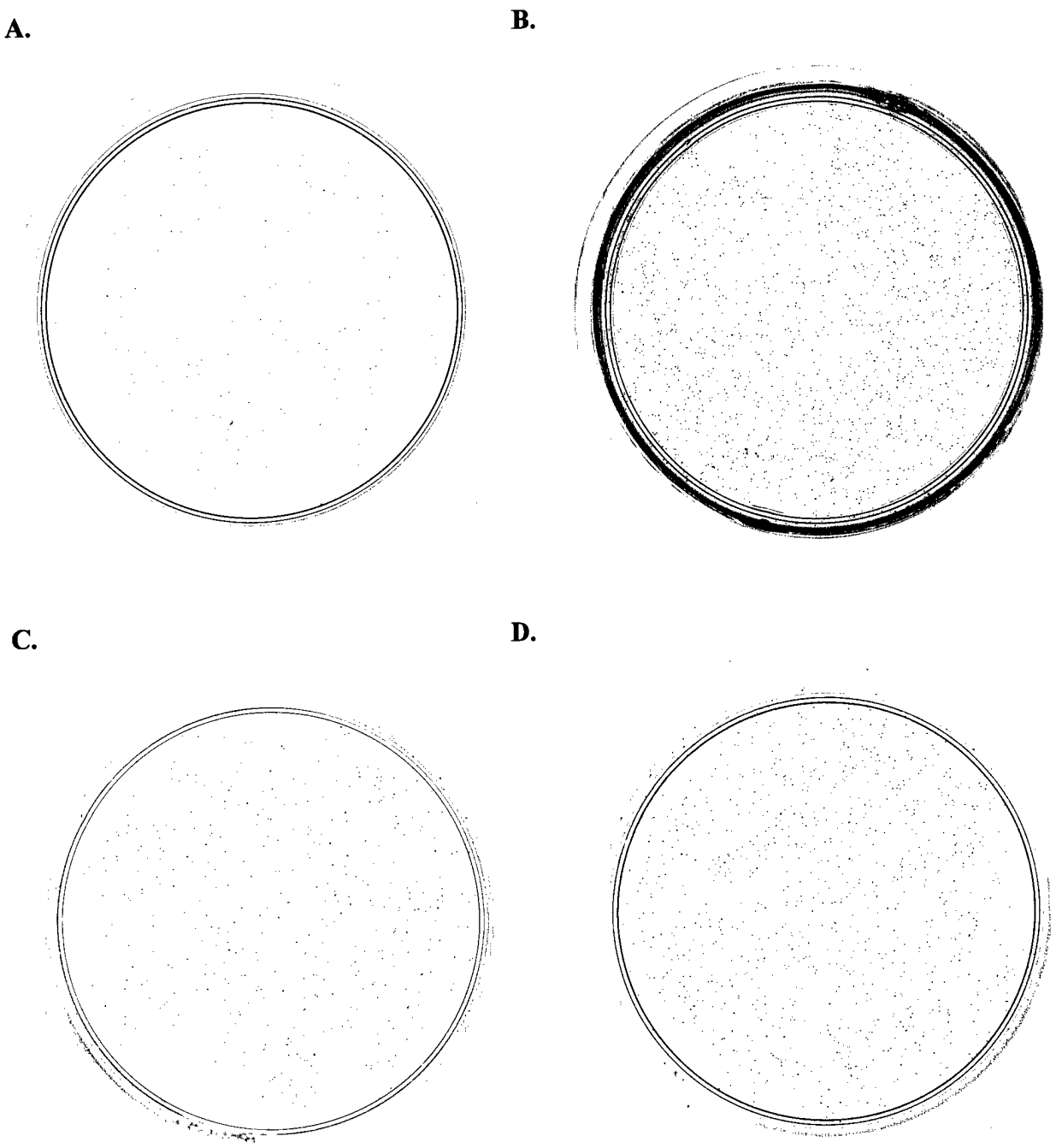

Figure 3: Over-expression of HMG-I or HMG-Y or both in MCF-7 cells leads to more colonies than those transfected with empty vector in soft agar. 1x 10^4 per petri dish (60 mm) of cells are seeded on 0.33% agar in 10%FCS culture medium. The cells were incubated at 37°C, and 5% CO_2 in humidified incubator for 14 days, followed by staining with p-iodonitrotetrazolium violet (INT) solution for 24 hrs. A: MCF-7 cells transfected with empty vector; B: MCF-7 cells transfected with HMG-I; C: MCF-7 cells transfected with HMG-Y; and D: MCF-7 cells co-transfected with HMG-I and HMG-Y.

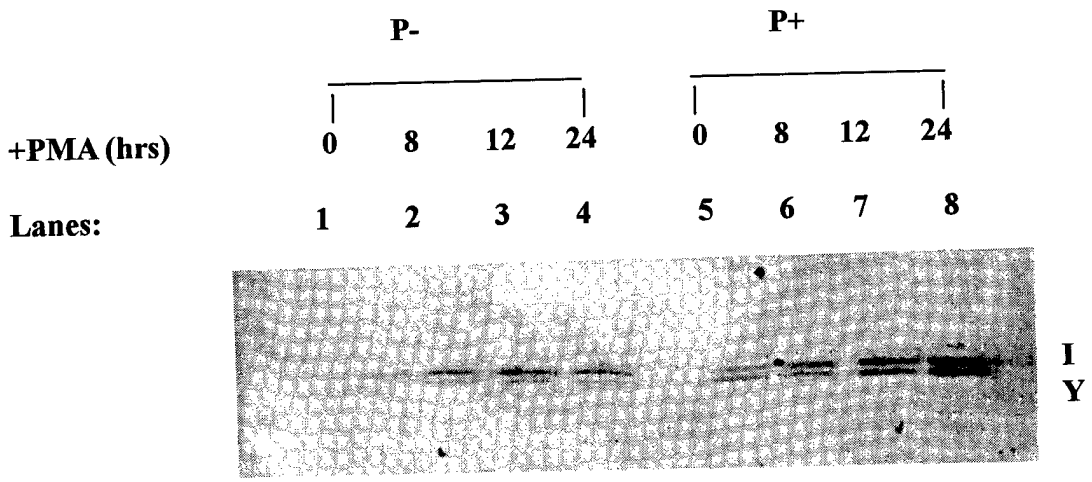

Figure 4: Different kinetics of HMG-I(Y) expression in P- and P+ murine epidermal cells in response to mitogen PMA stimulation. P- and P+ cells were treated with 10 ng/ml PMA for different time courses. Total cellular proteins from P- and P+ were isolated, run on SDS-PAGE, and then transfered onto cellulose membrane, followed by Western blot with a polyclonal antibody against human HMG-I(Y). Lanes 1-4 are from P- cells and Lanes 5-8 from P+ cells. Lanes 1, 5: without PMA treatment; Lanes 2, 6: with PMA treatment for 8 hrs; Lanes 3, 7: with PMA treatment for 12 hrs; and Lanes 4, 8: with PMA treatment for 24 hrs.

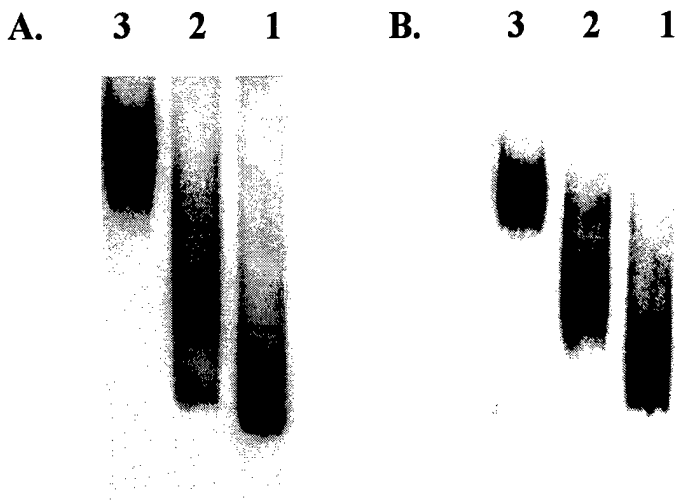

Figure 5: HMG-I(Y) proteins specifically bind to promoter regions of stromelysin (MMP-3). EMSA assay was performed at different regions of stromelysin 5' promoter. Panel A: Nucleotides # -754 to -210; Lanes 1-3 contain 0, 5.6ng and 28ng of HMG-I(Y) proteins respectively. Panel B: Nts. # -210 to -1: Lanes as in panel A.

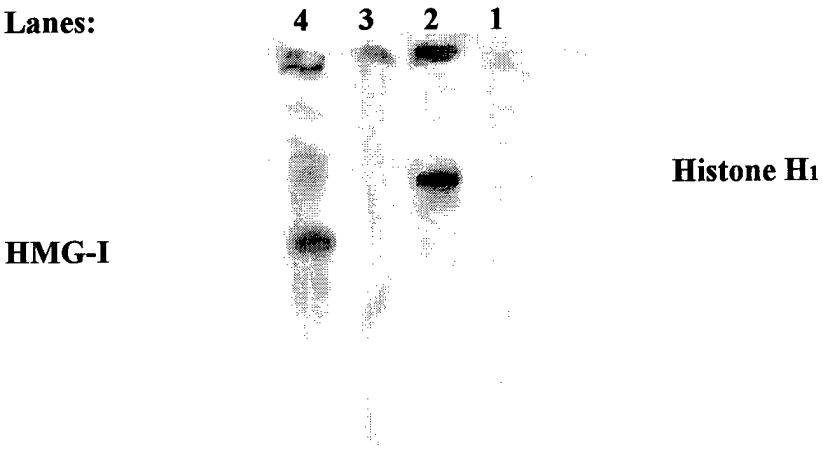

Figure 6: Phosphorylation of recombinant HMG-I protein by PKC in vitro. Recombinant HMG-I protein (5μg) was incubated with ^{32}P-labeled ATP and rat brain PKC (Calbiochem, San Diego, CA) for 2 min at 30°C according to manufacturer's protocol. The reaction was stopped by 25% TCA. The samples were run on 15% SDS-PAGE gel. The gel was scanned by Phospho-Image. Lanes 1, 3: without substrate. Lane 2: with Histone H1 substrate as positive control; and lane 4: with HMG-I substrate.

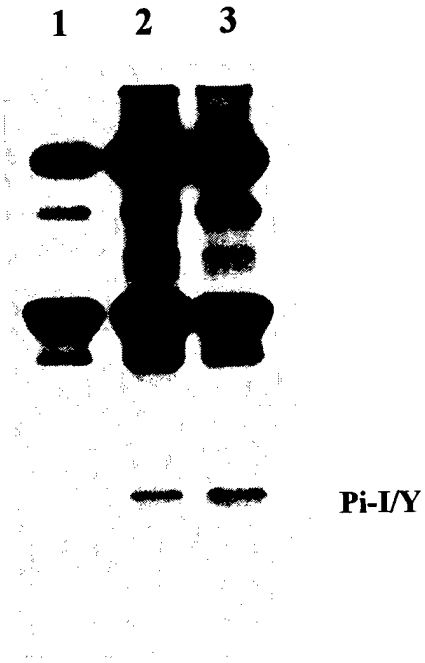

Figure 7: Rapid mitogenic induction of HMG-I(Y) phosphorylation in human breast Hs578T cells following stimulation with PMA/ionomycin. Hs578T cells were incubated in the presence of ^{32}P-labeled ATP for different time course. HMG-I(Y) proteins, which were isolated from the metabolic labeled Hs578T cells, were run on 15% SDS-PAGE gel and exposed to X-ray film. Lane 1, without PMA/ionomycin stimulation; Lanes 2 and 3: with PMA/ionomycin stimulation for 30 and 60 min respectively.

CONCLUSIONS

In the first year of research, considerable progress has been made according to the statement of work in the original grant as well as progress in one additional area that has opened up as a result of these findings. In brief, mammalian expression vectors which contain sense, antisense or dominant negative human HMG-I(Y) genes have been constructed, characterized and readied for further studies on tumor metastatsis and regulation of down-stream gene expression. The preliminary results of soft agar analysis indicated that over-expression of HMG-I(Y) in the human breast cancer cell line MCF-7 leads to higher tumorgenic potential. Furthermore, HMG-I(Y) proteins can physically interact with promoter regions of the matix metalloprotease MMP-3 gene, which is associated with tumor metastasis. All these results agree with the hypothesis in the original proposal. In addition, new findings have been obtained that HMG-I(Y) proteins can be phosphorylated by protein kinase C in vitro and phosphorylation of HMG-I(Y) proteins can be induced by mitogen PMA in the human breast cancer cell line Hs578T in vivo. These results indicated that HMG-I(Y) proteins could be the substrate of PKC and may play an important role in tumor metastasis.

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